(54) Title: A METHOD FOR SELECTIVE REMOVAL OF A SUBSTANCE FROM SAMPLES CONTAINING COMPOUNDS HAVING NUCLEIC ACID STRUCTURE

(57) Abstract: A method for purifying a desired substance comprising nucleic acid structure by separating substance (I) from substance (II), one of which is the desired substance, both of which have affinity for the same ligand structure, and substance (I) is smaller than substance (II). The method comprises: (i) providing substances I and II in a liquid; (ii) contacting the liquid with an adsorbent which selectively adsorbs substance I; (iii) recovering the desired substance; The adsorbent has (a) an interior part which carries a ligand structure that is capable of binding to substances I and II, and is accessible to substance I, and (b) an outer surface layer that does not adsorb substance II, and is more easily penetrated by substance I than by substance II. The use of certain anion exchangers funtionalized with a plurality of chargeable amine groups for the removal and/or purification of nucleic acid vectors. The matrices used should have (A) an increased elution ionic strength for at least one of a selection of standard proteins compared to a reference anion exchanger, or (B) have a hydroxy or amino nitrogen at a distance of 2-3 carbons from the amine groups.

A METHOD FOR SELECTIVE REMOVAL OF A SUBSTANCE FROM SAMPLES CONTAINING COMPOUNDS HAVING NUCLEIC ACID STRUCTURE.

Technical background.

The present invention concerns a method for purification of a desired substance comprising nucleic acid structure and comprises that a liquid sample containing a first substance (I) and a second substance (II) is contacted with a separation medium to which substance I has a stronger tendency to partition compared to substance II.

After the partitioning step, substance I is recovered from the adsorbent and/or substance II from the liquid, depending on which of them is to be purified. Finally, either or both of the substances may be further purified. For substances having nucleic acid structure two main principles have previously been used:

1. The separation medium has a firmly attached ligand structure to which substances I and II have different abilities to become bound to or desorbed from. Typically the ligand structure is an anion exchange group and the separation is based on ion exchange, e.g. ion exchange chromatography (IEC). Some basic publications are Cohn W.E. (in: Nucleic acids, Vol 1 pp 211-241 (1955), Chargaff & Davidsson (eds), Academic Press, New York); Hall et al (J. Mol. Biol. 6 (1963) 115-127); Bendich et al (J. Am. Chem. Soc. 77 (1955) 3671-3673); and Taussig et al (J. Chromatog. 24 (1957) 448-449).

2. The separation medium has a pore size permitting easier transport of substance I than of substance II within the pores. The separation is performed as a gel filtration (GF). Some basic articles are Hjertén (Biochim. Biophys. Acta 79 (1964) 393-); and Bengtsson et al (Biochim. Biophys. Acta 119 (1967) 399-); Öberg et al (Arch. Biochem. Biophys. 119 (1967) 504-509); and Loeb (Biochim. Biophys. Acta 157 (1968) 424-426).

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Separation media which have an interior part and an outer surface layer with different separation functionalities (e.g. anion exchange groups and no anion exchanging groups, respectively) have been previously described and suggested for the separation of proteins, nucleic acids, carbohydrates, lipids etc. See WO 9839094 (Amersham Pharmacia Biotech AB) and WO 9839364 (Amersham Pharmacia Biotech AB). None of these publications discloses how to use separation media in which there are layers of different functionalities for purifying nucleic acids in order to overcome the disadvantages discussed below. Purification of nucleic acid vectors such as plasmids, virus and the like and specific problems associated therewith are not discussed.

Some disadvantages of previous techniques.

Despite the innumerable reports published in this area during the past 30 years, it still remains a difficult task to separate negatively charged nucleic acids from each other and from other negatively charged components such as proteins.

This is partly due to the fact that the focus has changed from laboratory to large-scale processes. Thus it has become important to have processes that give high yields and high purity in a minimum of process steps in order to minimise production costs. Examples are various kinds of antisense drugs comprising synthetic oligonucleotides, recombinantly produced nucleic acids, such as nucleic acid vectors including viruses and plasmids, and recombinant proteins.

For compounds that comprise nucleic acid structure, individual process steps might increase the risk for conformational changes and irreversible denaturation/degradation, i.e. formation of contaminants, which are difficult to remove. This applies particularly to nucleic acids vectors, for instance plasmids. Covalently closed circular (CCC) plasmids

(supercoiled), for instance, may easily be transformed to the open circular form, which shows a lowered efficacy for therapy.

Substances having nucleic acid structures bind strongly to anion exchangers and desorption often require conditions that can be harmful for the product, in particular nucleic acid vectors such as plasmids. It would be beneficial to use anion exchange material that combine strong binding, high capacity with mild conditions for desorption.

Objectives of the invention.

A first objective is to provide methods for the purification of substances comprising nucliec acid structure, which methods are improved with respect to (a) simplicity of operation, (b) increased purity and yield of a desired substance and (c) a reduction of the number of steps involved. A sub-objective is to accomplish the same kind of improvements in relation to nucleic acid vectors such as plasmids and viruses.

A second objective is to minimise denaturation due to adsorption-desorption steps and other steps. A sub-objective is to minimise conformational changes and the risk for transformation of covalently closed circular vectors (CCC) to open circular vectors (OC). Another sub-objective is to provide modifications that will avoid adsorption/desorption of the final product or will enable desorption to take place under mild conditions, in particular for nucleic acid vectors.

A third objective is to provide improved methods facilitating the manufacture of plasmid preparations to be used as in vivo therapeutics. This means that the methods concerned shall give plasmid preparations that are of a therapeutically acceptable purity by which often is meant: CCC/OC > 80% preferably > 95%; RNA < 1% preferably < 0.6%; endotoxin <40 EU/mg; chromosomal DNA < 1% preferably < 0.6%; proteins < 1 µg/mg (preferentially

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proteins heterologous to the patient to be treated). All percentages are in w/w.

The invention.

5 We have now discovered that the separation methods for the purification of nucleic acids described in the introductory part can be improved if the adsorbent carries a shielding layer (lock, lid) which hinders passage of substance II into the interior part of the adsorbent matrix.

10

We have also discovered that, in particular for nucleic acid vectors, there are advantages if the anion exchange ligand is selected to provide

(a)   an enhanced binding via a mixed mode interaction, for
15   instance involving hydrogen-bonding or other electron donor-acceptor interactions combined with a charge-charge interaction and/or

(b)   milder desorption conditions by permitting decharging of the anion exchange ligands by a pH-switch (increase in pH)
20   at moderate alkaline pH-values.

A first aspect of the invention is a method for purifying a substance comprising nucleic acid structure from a substance, which has a different size but affinity for the same ligand
25 structure as the substance to be purified. In other words the method means separation of two substances from each other (substance I and substance II) which differ in size but have affinity for a common ligand structure.

30 The method thus comprises the steps of:
    i)   providing substances I and II in a liquid (sample);
    ii)   contacting the liquid with an adsorbent which has a high selectivity for adsorbing substance I compared to substance II;
35 iii) recovering the desired substance from the adsorbent as substance I and/or from the aqueous liquid as substance II;

If necessary, either or both [5] of the substances recovered in step (iii) is further purified.

The characteristic features of the inventive method are that
5 the separation medium used has
(a)   an interior part which

- carries a ligand structure which is capable of binding to both substances I and II, and
- is accessible to substance I, and

10 (b)   an outer surface layer that does not substantially adsorb substance II, and is easier penetrated by substance I than by substance II.

This means that the outer surface layer is accessible to
15 substances in the sample by convective mass transport, and that the interior part of the matrix is only accessible via diffusive mass transport. The outer surface layer may thus be considered as a border-layer limiting a convective environment from a diffusive environment.
20

The outer surface layer may be located to the outer surface of porous particles or to the surface of macropores within particles or within monoliths comprising both macropores and micropores. The pores, at least in the outer surface layer,
25 have a molecular size cut-off value for influx of compounds corresponding to an apparent molecular size between the apparent molecular sizes (hydrodynamic radius) for substance I and substance II, respectively. This typically means that the pores in the outer surface layer are < 1 μm. The interior part
30 may have pores with molecular cut-off values that are the same as pores in the outer surface layer, or have pores that are larger or smaller than these pores. The interior part may also contain a combination of these pore sizes.

35 The expression "carries a ligand structure which is capable of binding to both substances I and II" means that each of the

substances is capable of binding to the ligand structure if they have had access to it. It follows that the difference in selectivity between substance I and substance II for binding to the bead is primarily caused by the pore size of the outer
5 surface layer and not by a difference in the affinity as such for the ligand structure.

The expression "is easily penetrated by substance I compared to substance II" means that substance I is transported
10 substantially faster through the outer surface layer than substance II. This includes that substance II is completely excluded from the outer layer.

The expression "an outer surface layer that does not
15 substantially adsorb substance II" means that at least the surface of the layer is essentially free from adsorptive ligand structures.

The outer surface layer may also contain repelling structures,
20 e.g. structures of the same charge as substance I and II; hydrophobic structures in case substance II has a hydrophilic character that is incompatible with hydrophobic structures, etc. Repelling structures may improve the selectivity in transport through the outer surface layer. See WO 9839364
25 (Amersham Pharmacia Biotech AB).

The sample.
The sample can be derived from different sources and prepared in various ways. It may be derived from a blood sample, tissue
30 sample, cultured cells etc. It may be in the form of a cell lysate. It may also be a processed sample that has undergone centrifugation, filtration, ultrafiltration, dialysis, precipitation etc for removing particulate matters, proteins, certain fractions of nucleic acids, concentration, desalting
35 etc. Thus, it is common practice to
    (a) precipitate sample proteins before capturing and/or fractionating nucleic acids on an adsorbent,

(b) precipitate RNA, if a particular DNA fraction is to be isolated,

(c) reduce the ionic strength by desalting and/or diluting in case the sample is to be applied to an ion exchanger etc.

Other methodologies may also be applied in order to remove disturbing substances. In many cases the sample to be used in the instant invention is essentially free of particulate matters. For purification of plasmids the contents of contaminants in the sample typically are: protein $\leq$ 30 mg/ml, RNA $\leq$ 25 mg/ml. Endotoxin content may be > 200 EU per ml, typically > 40 000 EU per ml. Relative to total nucleic acid content, the plasmid may be present in quantities $\geq$ 3 % (w/w). Depending on the objective for a particular purification process and the starting material, the levels may be significantly lower. Similar values apply in case the desired substance is some other kind of nucleic acid vector, for instance a virus.

The sample typically is aqueous.

Substances I and II and apparent molecular size cut-off value. At least one of substances I and II has nucleic acid structure. The remaining substance may be some other compound as long as it comprises a structure that also is capable of binding to the ligand structure used. This means that the other substance may be a protein/polypeptide, an endotoxin, or a lipid, a detergent, a cell or a part thereof etc. Either or both of the substances may be a complex or conglomerate in which one or more components comprise nucleic acid structure while one or more other components comprise other structures. In important variants of the invention both substances I and II comprise nucleic acid structures (oligo- or polynucleotide structure). acid structure (oligo- or polynucleotide structure). Each of substance I or II may be mixtures of compounds.

Specific examples of substances, which have nucleic acid structure, are native and synthetic DNA and RNA including fragments and derivatives thereof having two or more nucleotides linked in sequence. Linear and circular forms of
5 nucleic acids, mRNA, tRNA, rRNA, genomic DNA etc are included. Still further examples are nucleic acid vectors such as viruses (including bacteriophages) and plasmids. Plasmids may be linear or circular. Circular forms include open circular (OC) forms and covalently closed circular (CCC) forms, i.e. supercoiled
10 forms.

The apparent molecular size of a substance is determined by (a) its molecular weight, and (b) its shape under the conditions applied. The apparent size may thus change upon change of pH,
15 ionic strength, type of salt and temperature. This is in particular true for biopolymers such as high molecular weight nucleic acids and proteins. Matching of pore sizes within the interior part and within the outer surface layer with apparent sizes of substances I and II is easily done by testing the
20 molecular size exclusion behaviour of different interior parts and locks. It will also be possible to draw conclusions from the size exclusion behaviour of the substances concerned on various size exclusion separation media. Common knowledge from size exclusion chromatography applies.
25

By properly setting the molecular size cut-off value of the outer surface layer, step (ii) of the present invention will facilitate separations of substances in a sample into two fractions, which contain substances of apparent sizes above,
30 respectively, below the molecular size cut-off value. One can thus envisage that the invention will render it possible to separate linear forms of DNA from circular forms of DNA, open circular forms from covalently closed circular forms, RNAs from plasmids, plasmids from genomic DNA, plasmids from plasmids,
35 plasmids from endotoxins etc. Typically the most useful molecular size cut-off values for the purification of plasmids will be in the interval corresponding to the apparent molecular

sizes for useful supercoiled plasmids, i.e. in the interval 1-10 kbp (kilo base pairs). This does not exclude that the cut-off value can be larger in case larger molecules are allowed to penetrate the interior part, for instance the interval may

5 correspond to nucleic acid vectors containing from 1 to 40 kbp.

In the preferred mode of the instant invention, the molecular size cut-off value of the outer surface layer is set so that the desired substance is retained in the liquid (substance II),

10 i.e. not transported to any significant extent into the interior part of the matrix. This principle has been found to be advantageous if the desired substance is substance II and is a nucleic acid vector, such as a virus or a plasmid. One of the main advantages is that the desired substance then does not

15 need to go through an adsorption/desorption process that may reduce yield and cause denaturation/degradation of the substance.

One can envisage that it will be possible to set molecular size

20 cut-off values that will make it possible to discriminate between high molecular weight genomic DNA and nucleic acid vectors. The smallest one (smallest apparent molecular size) will be bound to the interior part of the matrix while the largest one will be retained in the liquid.

25

Ligand structures

The ligand structure (ligand) as such shall have affinity for both substances I and II. Since at least one of the substances comprises a nucleic acid structure, the most apparent ligand

30 structures contain positively charged groups (anion exchanging groups). Anion exchanging groups in principle bind to any negatively charged species. Therefore, these kinds of ligand structures may be used in the instant invention for separating any negatively charged species from a substance comprising

35 nucleic acid structure. The only demand is that the difference in apparent molecular size shall be sufficiently large.

One and the same matrix may contain two or more different ligands, for instance anion exchange ligands.

Illustrative examples of anion exchanging groups are primary,
5 secondary, tertiary and quaternary ammonium groups that are linked via a spacer to a base matrix. Illustrative examples are $-N^+R_1R_2R_3$ in which R_{1-3} are hydrogen and/or hydrocarbon groups. The spacer is attached to the free valence of the $-N^+R_1R_2R_3$ group. The carbon chains in R_{1-3} may be interrupted at one or
10 more location by an ether oxygen (-O-) or a thioether sulphur (-S-) or a secondary, tertiary or quaternary ammonium group ($-N^+R_4R_5-$). The carbon chains may also be substituted by one or more $-OR_6$ or primary, secondary, tertiary or quaternary ammonium group ($-N^+R_7R_8R_9$) in which R_{4-9} are hydrogen or
15 hydrocarbon groups. The groups R_{1-9} may be identical or different. Hydrocarbon groups can be saturated, unsaturated or aromatic, and/or linear, branched or cyclic. R_{1-9} is typically selected amongst hydrogen or C_{1-10}, preferably C_{1-6}, hydrocarbon groups that preferably are alkyl groups. R_{1-9} may pair-wise, if
20 appropriate, form five- or six-membered rings including the atom(s) to which the involved R groups are attached.

The preferred anion exchange ligands provide mixed mode interaction with the substance to be bound and/or allow for
25 decharging by a pH-switch (increase in pH) at moderate alkaline pH-values. The ability of decharging means that the anion exchange ligands comprise primary, secondary and tertiary ammonium groups, with preference for those having pKa $\leq$ 10.5 or $\leq$ 10.0, i.e. typical primary or secondary ammonium groups. In
30 the variants believed to be most preferred and as reduced to practice in the experimental part, essentially all anion exchange groups should comply with this criterion.

The term "the anion exchange ligand provides mixed mode
35 interaction with the substance to be bound" refers to a ligand that is capable of providing at least two different, but co-

operative, sites which interact with the substance to be bound. One of these sites gives an attractive type of charge-charge interaction between the ligand and the substance of interest. The second site typically gives electron donor-acceptor interaction including hydrogen-bonding.

Electron donor-acceptor interactions mean that an electronegative atom with a free pair of electrons acts as a donor and bind to an electron-deficient atom that acts as an acceptor for the electron pair of the donor. See Karger et al., An Introduction into Separation Science, John Wiley & Sons (1973) page 42. Illustrative examples of donor atoms/groups are:

(a)   oxygen with a free pair of electrons, such as in hydroxy, ethers, carbonyls, and esters (-O- and -CO-O-) and amides,

(b)   sulphur with a free electron pair, such as in thioethers (-S-),

(c)   nitrogen with a free pair of electron, such as in amines, amides including sulphone amides], cyano,

(d)   halo (fluorine, chlorine, bromine and iodine), and

(e)   sp- and sp^2-hybridised carbons.

Typical acceptor atoms/groups are electron deficient atoms or groups, such as metal ions, cyano, nitrogen in nitro etc, and include a hydrogen bound to an electronegative atom such as HO- in hydroxy and carboxy, -NH- in amides and amines, HS- in thiol etc.

The distance between the donor or acceptor atom/group and the positively charged atom is typically 1-7 atoms, with preference for 2, 3, 4 and 5 atoms.

Examples of suitable anion exchange ligand structures may be found amongst those that contain primary, secondary and tertiary ammonium groups, typically containing no aromatic or unsaturated structures. Particularly preferred groups have one, two or more hydroxyl group or a primary, secondary or tertiary

amino nitrogen on at least one carbon atom that is located at a distance of 2 or 3 atoms away from the amino nitrogen of the ammonium group. These 2 or 3 atoms are typically sp^3-hybridised carbon atoms. One or more of these hydroxyl groups and amino nitrogens may or may not be present in the spacer. See also WO 9729825 (Amersham Pharmacia Biotech AB, = US 6,090,288) which is hereby incorporated by reference. Such exemplary ligands structures, inclusive the ending of the spacer (bold) are:

$-CHOHCH_2NH(CH_2)_3NH(CH_2)_3NH_2$ $-CHOHCH_2NHCH_2CH_2CH_3$

$-CHOHCH_2NHCH_2CH_2NHCH_2CH_2NHCH_2CH_2NH_2$ $-CHOHCH_2NH_2$

$-CHOHCH_2N[C(CH_2OH)_3](CH_2)_3NHC(CH_2OH)_3$ $-CHOHCH_2NHCH(CH_2OH)_2$

$-CHOHCH_2NHCH_2(CHOH)_4CH_2OH$ $-CHOHCH_2NHC(CH_2OH)_3$

$-CHOHCH_2NHC(CH_3)(CH_2OH)_2$ $-CHOHCH_2N[(CH_2)_3NH_2]_2$

$-CHOHCH_2NHCH_2CHOHCH_2OH$ $-CHOHCH_2NH[C(CH_3)_3]$

$-CHOHCH_2NHCH_2CH_2OH$ $-CHOHCH_2N(CH_2CH_2OH)_2$

$$-CHOHCH_2NH-\underset{\diagdown O - CHOH}{\overset{CHOHCHOH}{\overset{\diagup \diagdown}{CH}}} CHCH_2OH$$
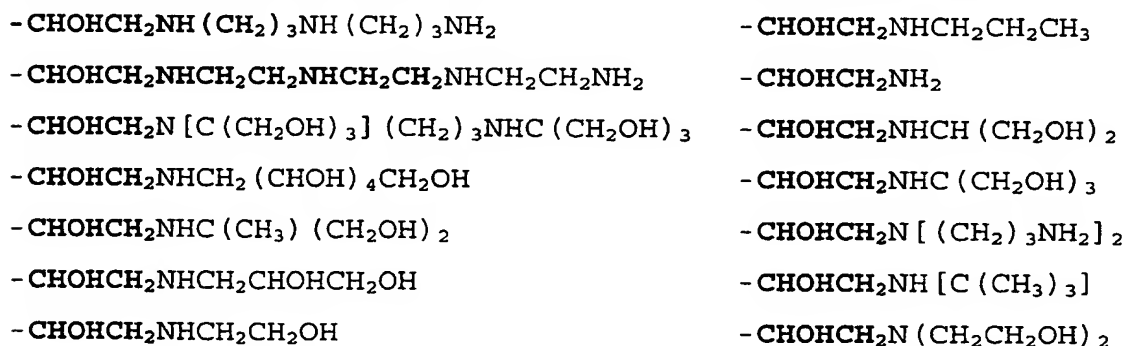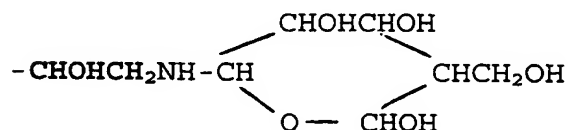
The ligand structures of particular interest are those that, when bound to matrix, can adsorb substances at increased ionic strength compared to a conventional reference anion exchanger. In most cases this means that the preferred anion exchangers will exhibit an increased elution ionic strength compared to a conventional reference anion exchanger. This can be expressed in such a way that the maximum elution ionic strength in the pH range 2-14 for an anion exchanger (I) carrying an ammonium ligand structure as defined above should be higher than, in preferred cases ≥ 125%, in many cases ≥ 140%, such as ≥ 200% of the required elution ionic strength for a quaternary anion exchanger (II) with the ion exchanging group $(CH_3)_3N^+-$ (= Q-group; the same matrix, the same coupling group from the quaternary nitrogen and in towards the matrix, the same level of ligand concentration as for the anion exchanger (I) and measured at the same pH) for desorption of at least one of the

proteins transferrin, ovalbumin 1, ovalbumin 2, β-lactoglobulin 1 and β-lactoglobulin 2. See WO 9729825 (Amersham Pharmacia Biotech AB).

5   According to another selection criterion suitable anion-exchangers may be found amongst those that have a maximal breakthrough capacity somewhere in the pH-interval 2-12 for at least one of the reference proteins: ovalbumin, conalbumin, bovine serum albumin, β-lactglobulin, α-lactalbumin, lyzozyme,

10 IgG, soybean trypsin inhibitor (STI) which is $\geq$ 200%, such as $\geq$ 300% or $\geq$ 500% or $\geq$ 1000% of the corresponding breakthrough capacity obtained for a Q-exchanger ($-CH_2CH(OH)CH_2N^+(CH_3)_3$. The support matrix, degree of substitution, counter-ion etc are essentially the same in the same sense as discussed above. The

15 reference anion-exchanger is Q Sepharose Fast Flow (Amersham Pharmacia Biotech AB, Uppsdala, Sweden). This reference anion-exchanger is a strong anion-exchanger whose ligand and spacer arm structure are:

$$-O-CH_2CHOHCH_2OCH_2CHOHCH_2N^+(CH_3)_3.$$
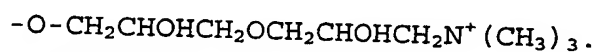
20 Its chloride ion capacity is 0.18-0.25 mmol/ml gel. The base matrix is epichlorohydrin cross-linked agarose in beaded form. The beads have diameters in the interval 45-165 μm. The exclusion limit for globular proteins is 4×10^6.

25 See further International Patent Applications (Amersham Pharmacia Biotech AB) based on SE application SE 9904197-2 with filing date November 22, 1999. These International Patent Applications are hereby incorporated by reference.

30 Alternative ligand structures may be selected amongst nucleic acid structures complementary to at least part of the nucleic acid structure of substance I. The complementarity should be sufficient for permitting hybridisation between the ligand structure and substances I under the binding conditions

35 applied. This kind of ligand structure requires that substance II also carries a nucleic acid structure that at least

partially is essentially the same as in substance II. Poly-U
and nucleic acid binding proteins are examples.

5 In case substances I and II also have other structures than
nucleic acid structures and other negatively charged groups,
ligand structures binding to these could also be used for
capturing substance I selectively by the separation medium
according to the instant invention.

10 The ligand structure is typically covalently linked to the
matrix via a spacer as known in the field. The spacer may be an
organic structure, which is hydrolytically stable under the pH
conditions normally utilized for anion exchange adsorption,
i.e. pH 2-14. The spacer typically lacks hydrolytically
15 unstable structures, such as silane, carboxylic acid ester
(-COO-) or carboxylic acid amide (-CONH-). The spacer is
preferably a linear, branched or cyclic saturated or
unsaturated hydrocarbon chain. The chain is optionally
interrupted at one or more locations by an ether oxygen (-O-)
20 or a thioether sulphur (-S-) and/or an amino nitrogen
($-N^+R_{10}R_{11}-$) or substituted by one or more $-N^+R_{12}R_{13}R_{14}$ groups or
$-OR_{15}$ groups. R_{10-15} are selected according to the same rules as
for the other R groups discussed above. The ligand structure
may also be bound non-covalently as long as the link is capable
25 of withstanding the conditions used for adsorption/desorption.

As discussed in WO 9729825 (Amersham Pharmacia Biotech AB)
insertion of primary, secondary or tertiary amine ligands is
easily done by methods not giving rise to any significant
30 amount of quaternary ammonium structures. This latter kind of
structure are not dechargeable by a simple pH-shift.

The interior part of the matrix
This part of the matrix is typically of the same type as
35 commonly utilized within affinity adsorption such as
chromatography. As discussed above the interior part may
comprise both macropores and micropores.

The interior part is preferably hydrophilic and in the form of a polymer, which is insoluble and more or less swellable in water. Hydrophilic polymers typically carry polar groups such as hydroxy, amino, carboxy, ester, ether of lower alkyls (such as $(-CH_2CH_2O-)_nH$, $(-CH_2CH(CH_3)O-)_nH$, and groups that are copolymerisates of ethylene oxide and propylene oxide (e.g. Pluronics®) (n is an integer > 0, for instance 1, 2, 3 up to 100). Hydrophobic polymers that have been derivatized to become hydrophilic are also included in this definition. Suitable polymers are polyhydroxy polymers, e.g. based on polysaccharides, such as agarose, dextran, cellulose, starch, pullulan, etc. and completely synthetic polymers, such as polyacrylic amide, polymethacrylic amide, poly(hydroxyalkyl vinyl ethers), poly(hydroxyalkylacrylates) and polymethacrylates (e.g. polyglycidylmethacrylate), polyvinylalcohols and polymers based on styrenes and divinylbenzenes, and copolymers in which two or more of the monomers corresponding to the above-mentioned polymers are included. Polymers, which are soluble in water, may be derivatized to become insoluble, e.g. by cross-linking and by coupling to an insoluble matrix via adsorption or covalent binding. Hydrophilic groups can be introduced on hydrophobic polymers (e.g. on copolymers of monovinyl and divinylbenzenes) by polymerization of monomers exhibiting groups which can be converted to OH, or by hydrophilization of the final polymer, e.g. by adsorption of suitable compounds, such as hydrophilic polymers.

The interior part can also be based on inorganic material, such as silica, zirconium oxide, graphite, tantalum oxide etc.

The interior part is preferably devoid of hydrolytically unstable groups, such as silan, ester, amide groups and groups present in silica as such.

In a particularly interesting embodiment of the present invention, the interior part is in the form of irregular or spherical beads with sizes in the range of 1-1000 μm, preferably 5-1000 μm.

The interior part may also be in the form of a porous monolith.

The ligand structures are introduced into the interior part by methods known in the field as suggested above under the heading "Ligand Structures".

The required degree of substitution for ligand structures (density of ligand structures) will depend on ligand type, kind of matrix, compound to be removed etc. Usually it is selected in the interval of 0.001-4 mmol/ml matrix, such as 0.01-1 mmol. For agarose-based matrices the density is usually within the range of 0.1-0.3 mmol/ml matrix. For dextran based matrices the interval the interval may be extended upwards to 0.5-0.6 mmol/ml matrix.

The ranges given in the preceding paragraph refer to the capacity for the matrix in fully protonated form to bind chloride ions. "ml matrix" refers to the matrix saturated with water. The outer surface layer is included in the matrix in calculating these ranges.

The outer surface layer
The outer surface layer must be penetrable by the liquid sample. For aqueous liquid this means that the outer surface layer should be built up of a hydrophilic polymer of the same kind as discussed for the interior part

There are different methodologies for creating the outer surface layer.

I.   Coating the surface of a naked form of a porous particle or the surfaces of macropores of particles or of a monolith

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which have both macropores and micropores with a hydrophilic polymer. The apparent molecular size of the hydrophilic polymer should be selected such that it cannot significantly penetrate the pores that are aimed at being part of the interior. Preferably the hydrophilic polymer comprises hydrophilic groups as discussed above, e.g. is a polyhydroxy polymer such as polysaccharides in soluble forms (dextran, agarose, starch, cellulose etc). The ligand structures may be introduced onto the interior part either before or after creation of the lock. The permeability for various substances of the outer surface layer produced in this way will be controlled by the concentration and size of the polymer in the solution used for coating. Subsequent to coating the outer surface layer may be stabilized by crosslinking within the layer as well as to the interior part. This methodology is described in detail in WO 9839094 (Amersham Pharmacia Biotech AB).

II. Starting from a naked hydrophilic base matrix of the type discussed under the heading Interior Part above and then specifically introducing the ligand structure into an interior part of the matrix leaving an outer surface layer devoid of ligand structure. It is preferred to select the porosity of the starting matrix such that substance I will have a facilitated transport compared to substance II within the matrix, i.e. the pore size of the interior part and the outer surface layer are essentially the same. This kind of methodology has been presented in WO 9839364 (Amersham Pharmacia Biotech AB).

The lock medium used in the present invention may be in the form of particles/beads that have densities higher or lower than the liquid (for instance by introducing one or more density-controlling particles per matrix particle). This kind of matrix is especially applicable in large-scale operations for fluidised or expanded bed chromatography as well as different batch-wise chromatography techniques in non-packed columns, e.g. simple batch adsorption in stirred tanks. These

kinds of techniques are described in WO 9218237 (Amersham Pharmacia Biotech AB) and WO 92/00799 (Kem-En-Tek/Upfront Chromatography) and can easily be adapted to the inventive concept by introducing a lock on the particles used.

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Process conditions

The conditions for running the inventive process are in principle the same as for conventional adsorption techniques, e.g. anion exchange chromatography.

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For positively charged ligand structures this means that the matrix is first equilibrated to a suitable pH where the ligand structures are positively charged and an ionic strength that is well below the maximum ionic strength permitted for adsorption. 15 This typically means that the ionic strength should be below the elution ionic strength for the particular combination of substance(s), anion exchanger and other conditions etc. The sample is then applied. After adsorption either or both of the liquid phase and the matrix are further processed with respect 20 to substances I and II, respectively. Desorption of substance I from the matrix is accomplished by increasing the ionic strength of the liquid in contact with the matrix until substance I is eluted. In particular in case the ligand structure is the protonated form of a primary, secondary or 25 tertiary amine group and/or substance I is a nucleic acid, desorption is preferably assisted by increasing the pH. An alternative method for desorption is to include a soluble ligand analogue in the liquid, i.e. a structure analogue that is able to compete with the ligand structure for binding to 30 substance I. The presence of structure-breaking compounds in the liquid may also assist desorption. This in particular may apply in case the ligand structure contains one or more hydroxyl group or amino group at a carbon atom at 2 or 3 atoms distance from a charged primary, secondary or tertiary nitrogen 35 of the ligand structure. Well-known structure breaking agents

are guanidine and urea. See also WO 9729825 (Amersham Pharmacia Biotech AB).

The above-mentioned desorption principles may be combined as
5 found appropriate for a particular ligand structure and substance I

Changes in the composition of the liquid in contact with the matrix can be made in order to accomplish desorption of
10 substance I either as a step-wise gradient or a continuous gradient with respect to pH and/or concentration of salt and/or other desorbing agents. If possible it is simplest to make the change in one step. Continuous gradients and stepwise gradients containing two or more steps have their primary use in case
15 substance I has been bound to the matrix together with one or more additional substances. In these cases the desorption gradient may be used for desorbing the substances during different conditions thereby improving the purity of recovered substance I.
20

As indicated above either substance I or II may be further purified, for instance by so called polishing and or intermediate purification steps. After desorption, substance I may be further purified by additional capture steps either for
25 capturing the desired substance or for capturing contaminants. If substance II is desired in purified form it may also be subjected to additional capturing step. The need for extra purification/polishing steps typically applies if the purity demand on the desired substance is high, such as for in vivo
30 therapeutics. Such additional steps may involve adsorbtion/desorption of substance I or II to/from an anion exchanger, a cation exchanger, a reverse phase matrix, a HIC matrix (hydrophobic interaction chromatography matrix) etc. Size exclusion chromatography and adsorption/desorption on
35 hydroxy apatite may also be used. There may also be one or more of the above-mentioned adsorption/desorption steps before the step, which utilizes a lock based affinity matrix.

For large-scale production of the desired substance as defined above it is of utmost importance to have selected matrix material, ligand and coupling chemistry that will permit desorption, regeneration and re-use of the adsorbent/separation medium. Re-use typically starts with regenerating and equilibrating the adsorbent after step (iii) whereafter the adsorbent is contacted as defined in step (ii) with a new batch of sample. The regeneration and equilibration is done as known in the field. In certain variants these two steps may coincide. This kind of cyclic use of the separation medium typically demands a cleaning step either before or after the regeneration step. A cleaning step may be present in each cycle, or every second, third, fourth, fifth etc cycle or whenever found appropriate.

A second separate aspect of the invention is the use of separation media, which carry the above-mentioned primary, secondary and tertiary ammonium groups and

(a) which are able to adsorb at an increased ionic strength as defined above and/or

(b) which have one, two or more hydroxyl groups and/or amino nitrogens at a distance of two or three sp^3-hybridised carbon atoms from the ammonium groups,

for the removal and/or purification of nucleic acid vectors.

In this aspect of the invention the ability to adsorb at an increased ionic strength, the kind of ligand and spacer, matrix features, such as porosity, matrix material, etc are as defined above. The matrix may be fully functionalized, or only functionalized in its interior as defined above. The amino nitrogens referred to are preferentially primary, secondary or tertiary amino nitrogens with sub-alternatives and preferences as discussed for the first aspect of the invention.

This separate aspect is based on our previous discovery that anion exchangers in which one, two or more hydroxyl groups

and/or amino nitrogens are present at a distance of two or three sp³-hybridised carbons from a positively charged amine nitrogen enhances binding of substances to the anion exchangers. See WO 97298725 (Amersham Pharmacia Biotech AB). We

5 have know recognized that this can give certain advantages when dealing with nucleic acid vectors. See above.

One variant of this aspect is the purification method defined in the first aspect.

10

In another variant the separation medium lacks the outer surface layer (including a lock). The base matrix carrying the ligand structure in this variant may be of the same construction as the interior part described above. This variant

15 means that the separation medium is used in a conventional capture step, for instance as described in WO 9916869 (Amersham Pharmacia Biotech AB). The vector plus contaminating species such as RNAs are selectively adsorbed and desorbed. The full process may contain additional purification steps as defined

20 above for the process utilizing a lock separation medium.

In both variants the sample which contains the nucleic acid vector may have been treated as known in the field in order to remove proteins and/or nucleic acids.

25

See for instance WO 9916869 (Amersham Pharmacia Biotech AB) and Ollivier and Stadler, Gene Therapy of Cancer (editors Walden et al), Plenum Press, New York (1998) 487-492 and GB patent application 9927904.4 and corresponding International patent

30 Application.

The invention is further defined in the appended claims. The invention will now be verified and illustrated with a number of patent examples.

35

E X P E R I M E N T A L   P A R T

SYNTHESIS

EXAMPLE 1. Anion exchanger in particle form with a lock on the particles.

5 **A. Allylated crosslinked agarose particles (allylated base matrix).** Cross-linked agarose (90 μm particles) prepared by reaction between epichlorohydrin and agarose in the presence of NaOH according to Porath et al (J. Chromatog. 60 (1971) 167-77 and US 3,959,251) was reacted with allylglycidyl ether with 10 NaOH as a base to a allyl level ($CH_2=CHCH_2OCH_2CHOHCH_2-$) of 0.18-0.30 mmole/ml). This base matrix has a porosity which is similar to Sepharose 4B FF (Amersham Pharmacia AB, Uppsala, Sweden).

15 **B. Introduction of a lock on allylated crosslinked agarose particles.** 25 g vacuum drained allylated particles from A with an allylic content of 0.29 mmol/ml gel was charged together with 0.6 g anhydrous sodium acetate and 50 ml de-ionized water in 100 ml beaker fitted with a propeller stirrer.
20 0.18 ml bromine was added drop-wise under rapid stirring.

The brominated gel was then washed with plenty of de-ionized water and vacuum drained on a glass filter funnel. Gel and water were charged in a three-necked round flask. The water was 25 added to a total weight of 50 g water and gel.

2 g Sodium hydroxide and 0.03 g sodium borohydride were then added and the temperature was raised to 60°C. After 21 h, 6 g thioglycerol was added in order to neutralise possible 30 unhydrolysed epoxides. The reaction mixture was stirred for another 4 h at 60°C. The reaction was stopped by washing the gel on a glass filter funnel with water. A small amount acetic acid was added directly in the glass filter funnel and the slurry was made slightly acidic. A last wash with plenty of de-35 ionized water was carried out.

The remaining allylic content was determined to 0.21 mmol/ml.

C. Introduction of anion exchange ligand on lock beads prepared from allylated crosslinked agarose particles. 15 ml vacuum drained particle from B above, 0.63 g anhydrous sodium acetate and 100 ml de-ionized water was charged in 250 ml beaker fitted with a propeller stirrer.

Bromine (0.20 ml) was added drop-wise under rapid stirring.

The gel was washed with plenty of water. After vacuum draining on a glass filter funnel the gel was charged in a three necked 100 ml round flask already containing 22.5 g TRIS ($NH_2C[CH_2OH]_3$) and 22.5 g water.

The reaction was carried out at 60°C over night 22.5 h.

The gel was then washed with a few bed volumes of water before pH was adjusted to about 7. Another washing step using plenty of water was carried out.

The material was sieved on a 45 µm sieve in order to get rid of small and crushed beads. The material left on the sieve was used as column packing in the chromatography experiments.

The total chloride ion capacity was determined to 0.08 (0.076) mmol/ml gel.

EXAMPLE 2. Reference matrix without lock (naked matrix) functionalized with Tris ligand.

A. Allylated crosslinked agarose particles (allylated base matrix). This base matrix was prepared in the same way as in Example 1A. The allyl-ligand density was determined to 0.26 mmol/ml matrix.

5

B. Coupling of Tris (tris(hydroxymethyl) amine). 10 ml vacuum drained allylated gel from example 2A, 1.2 g sodium sulfate and 50 ml distilled water was mixed in 100 ml beaker fitted with a propeller stirrer. Bromine was added dropvise under rapid

10 stirring until the slurry turned permanently yellow.

The gel was washed with plenty of water. After vacuum draining on a glass filter funnel the gel was transferred to a three necked 25 ml Bellco flask with a hanging magnetic stirrer which

15 already contained 15 g Tris and 15 g distilled water. The reaction was carried out at 60°C over night.

The pH of the reaction mixture was adjusted to 7 with dilute hydrochloric acid. A washing step using plenty of water (more

20 than 100 ml) was carried out.

The final product had a ligand density (Ion Exchange Capacity) was 0.17 mmol/ml

25 **CHROMATOGRAPHY**

EXAMPLE 3. Chromatographic experiments with purified plasmids.

I. Materials

Separation media: Lock particles according to example 1 (separation medium A) and particles without lock according

30   to example 2 (separation medium B).

Plasmid preparation: E. coli cells harbouring plasmid PXL 2784 (size = 6.3 kbp) were lysed according to the standard alkaline lysis method of Birnboim (Birnboim et al., Nucleic Acids Res. 7 (1979) 1513-1523; and Birnboim, Meth. Enzymol.

35   100 (1983) 243-255). The sample was not treated with RNAse.

The purified plasmid PXL 3096 (2.5 kbp) was purified by using
    essentially hydroxy apatite chromatography while PXL 2784
    (6.3 kbp) was prepared here in Uppsala using the Qiagen Kit
    (Qiagen) which meant RNAse treatment.

5 Equilibration buffer (A): 10 mM Tris-HCl, 1 mM EDTA, pH 8.0
    Elution buffer (B): 1 M NaCl in Buffer A, pH 8.0

II. Chromatography

A column (HR 10/3 (Amersham Pharmacia Biotech AB, Uppsala,
10 Sweden) containing separation medium A or B (bed volume 2.4 ml)
was equilibrated at a flow rate of 30 cm/h. Then freshly
prepared, and clarified, alkaline lysate (2 ml, containing
about 50-80 μg of plasmid DNA) was applied. The column was
then washed with: (i) 3 CV of the equilibration buffer to elute
15 unbound material and, (ii) 3 CV of Buffer B to elute bound
material. Fractions were pooled directly as they emerged from
the column. When deemed necessary, the column was washed with 2
CV of 1 M NaOH followed with 3 CV of water.

20 ## III. Electrophoretic analysis

This was performed on a 1% agarose gel (for nucleic acids)
using a "sub-marine" electrophoresis assembly (GNA-100, 8
wells, 5 x 1 mm) and EPS 500/400 power supply (Amersham
Pharmacia Biotech AB). A standard TBE buffer was used for
25 electrophresis. The gels were stained with ethidium bromide and
visualized by a UV lamp.

RESULTS:

Separation medium A. Chromatography of purified plasmids on
30 **anion exchange particles with a lock.** About 70 μg each of the
purified plasmids PXL 3096 (2.5 kbp) and PXL 2784 (6.3 kbp)
were chromatographed on the lock particles according to the
procedure outlined above. The results showed that none of these
2 plasmids was bound to the column indicating that the porosity
35 of the lock or polymer shield is such that diffusion of these
macromolecules into the charged outer or inner surfaces of the

26

anion-exchange particles is blocked. In effect, the plasmids are eluted in the void volume of the column and the lock medium acts as a passive molecular sieve.

5 **Separation medium B. Chromatography of purified plasmids on anion exchange particles without a lock.** The above experiment was repeated using particles without a lock according to example 2 under identical experimental conditions. The results showed that both plasmids are bound to this separation medium. 10 This is because the plasmids have access to at least the charged outer surfaces of the anion-exchanger. The results also showed that the step-wise elution of the bound plasmids leads to their separation into at least 2 sub-fractions. The nature of these sub-fractions is not yet established and will be a 15 topic for future investigations.

These results thus provide strong proof that the lock concept in media construction works in "real life situations" to solve one of the most difficult separation problems in biochemistry. 20

EXAMPLE <u>4. Chromatography of clarifed alkaline lysate (CAL) on a lock medium.</u>

25 This has been performed under varying experimental conditions. For purposes of clarity, the results obtained will be presented in 3 separate sections.

I.   Effect of de-salting
30 2 ml of the clarified alkaline lysate (CAL) (sample A) containing the 6.3 kb plasmid was applied to the column without any further treatment and eluted according to the procedure outlined under "Experimental" in example 3. The experiment was repeated using 10 ml of de-salted CAL (which is equivalent to 35 ca. 5 ml of the crude plasmid extract due to dilution during de-salting) (sample B). Desalting was performed on a PM10

membrane (Amicon, U.S.A.) in a stirred cell using nitrogen gas
to generate constant pressure. The material used was the same
as in example 3 except for the equilibration and elution
buffers.

5   <u>Equilibration buffer (A)</u>: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0
       <u>Elution buffer (B)</u>: 1 M NaCl in buffer (A), pH 8.0

Results:

The plasmid was in both cases eluted in the unbound fraction.
10 The bound fraction eluted as a broad peak (5-10 column volumes
(CV)). The recovery in A260 was about 80%. The gel
electrophoretic pattern obtained as described in example 3.III
showed in both cases that the unbound fractions contained
exclusively the plasmid while the bound fraction contained the
15 RNA impurities. The unbound fraction for sample B showed a
streaking band apparently because the plasmid might have been
damaged when it was desalted. The unbound fraction for sample A
seemed to contain a small amount of RNA possibly due to the
high salt concentration in the sample resulting in a decreased
20 adsorption capacity for the RNA. The following conclusions are
consistent with the results obtained above:
   1. The unbound fraction contains the plasmid DNA while the
      bound fraction contains RNA.
   2. The bound fraction is eluted in a broad peak. The broadness
25   of the peak may be due to a diffusion barrier created by the
      lock.
   3. De-salting of the sample might be necessary to increase the
      adsorption capacity of the medium for RNA and other
      impurities.
30 4. The recovery in A_{260} is ca. 80 % indicating that some of the
      impurities are strongly bound and may require a cleaning
      step, e.g. washing with 1 M NaOH, to be completely eluted.

II. Effect of pH

35 Anion exchange media containing the ligand used in Example 1
has its "optimum binding strength" for most anionic proteins at

ca. pH 5.5-7.0. It is also virtually uncharged at around pH 9.0. See WO 9729825. One would therefore expect an efficient de-sorption of bound molecules from this adsorbent at approx. pH 9.0 or higher pH. The following experiment was therefore

5 performed to establish/reject the validity of the above findings to plasmid DNAs. Except for the sample and the buffers used the chromatographic experiment was the same as in example 3.III.

Sample: Clarified crude alkaline lysate (pH = 5.3). Not de-

10   salted.

Equilibration buffer (A): 50 mM sodium acetate, 1 mM EDTA, pH
           5.5

Elution buffer (B): 10 mM Tris-HCl, 1 mM EDTA, pH 9.1

15 **Results**:

Based on the chromatogram and the gel electrophoretic pattern it was that the plasmid eluted similarly to previous experiments in the unbound fraction and RNA in the bound fraction (in about 10 CV). The recovery in A_{260} is about 80%.

20

Conclusions

1. As expected, the impurities were bound more strongly at pH 5.5 than at pH 8.0 just as in the case with anionic proteins. This might indicate that the separation medium

25   used has a higher adsorption capacity for RNA (and possibly other impurities) at pH 5.5 and possibly even at pH 6.5.

2. The slower de-sorption kinetics might reflect a slow titration of the ion-exchanger from pH 5.5 to pH 9. If this is the case, and since the bound fraction comprises unwanted

30   impurities, one can speed up the process by washing the column with 0.5 M NaOH. This has the added advantage that even strongly bound solutes might be eluted.

III.   **Effect of the unfunctionalised outer surface layer (lock).**

The material and the procedure in this experiment were similar to example 3.

Separation medium: According to example 2, i.e. medium without lock.

Sample: 5 ml of the clarified alkaline lysate (pH adjusted to 6.4). Not de-salted.

Equilibration buffer (A): 20 mM sodium phosphate, 1 mM EDTA, pH 6.4

Elution buffer (B): 20 mM Tris-HCl, 1 mM EDTA, 0.5 M NaCl, pH 9.2

Procedure: See example 3.

The bound and unbound fractions were analysed by gel electrophoresis as described in example 3.

Results:

The unbound fraction eluted as a broad peak in about 15 CV. The unbound fraction contained exclusively plasmid DNA while the bound fraction contained both plasmid DNA and RNA.

C L A I M S

1. A method for purifying a desired substance comprising
   nucleic acid structure by separating from each other
5 substance (I) from substance (II), one of which is the
   desired substance, both of which have affinity for the
   same ligand structure, and substance (I) has a smaller
   size than substance (II),
   said method comprises the steps of:
10   (i)   providing substances I and II in a liquid (sample);
     (ii) contacting the liquid with an adsorbent which has a
           high selectivity for adsorbing substance I compared
           to substance II;
     (iii) recovering the desired substance from the adsorbent
15   as substance I or from the aqueous liquid as
           substance II;
     (iv) further purifying, if necessary, the substance
           recovered in step (iii);
   characterized in that the adsorbent has
20 (a) an interior part which
     • carries a ligand structure that is capable of binding
       to substances I and II, and
     • is accessible to substance I, and
   (b) an outer surface layer that does not substantially adsorb
25   substance II, and is more easily penetrated by substance
      I than by substance II.

2. The method of claim 1, **characterized** in that the outer
   surface layer is penetrable by substance I but not by
30   substance II.

3. The method of any of claims 1-2, **characterized** in that the
   ligand structure comprises a positively charged group.

4. The method of claim 3, **characterized** in that the positively charged group is a primary, secondary or tertiary ammonium groups.

5. 5. The method of any of claims 1-4, **characterized** in that substance I and the ligand structure comprises nucleic acid structures that at least partially are complementary thereby permitting hybridisation to each other during step (ii).

10

6. The method of any of claims 1-5, **characterized** in that the outer surface layer is essentially free of ligand structures.

15 7. The method according to any of claims 1-6, **characterized** in that substance II is the desired substance.

8. The method according to any of claims 1-7, **characterized** in that substance I is the desired substance.

20

9. The method of any of claims 1-8, **characterized** in that both substances I and II comprise a nucleic acid structure.

10. The method of any of claims 1-9, **characterized** in that the
25 substance to be purified is a nucleic acid vector.

11. The method of claim 10, **characterized** in that the vector is a plasmid.

30 12. The method of claim 10, **characterized** in that the vector is a virus.

13. The use of an anion exchanger in which the anion exchange ligands are primary, secondary or tertiary ammonium groups
35 and which exhibits an increased elution ionic strength for

32

the removal and/or purification of a nucleic acid vector from an aqueous sample,

said increased elution ionic strength meaning that the maximum elution ionic strength in the pH range 2-14 for the

5   anion exchanger is higher than, in preferred cases $\geq$ 125%, in many cases $\geq$ 140%, such as $\geq$ 200% of the required elution ionic strength for a quaternary anion exchanger (II) with the ion exchanging group $(CH_3)_3N^+-$ (= Q-group); the same matrix, the same coupling group from the

10   quaternary nitrogen and in towards the matrix, the same level of ligand as for the anion exchanger (I) and measured at the same pH) for desorption of at least one of the proteins transferrin, ovalbumin 1, ovalbumin 2, β-lactoglobulin 1 and β-lactoglobulin 2.

15

14. The use of an anion exchanger which carries a plurality of primary, secondary or tertiary ammonium groups for the removal and/or purification of a nucleic acid vector from an aqueous sample, said ammonium groups having one, two or

20   more hydroxyl groups and/or amino nitrogens at a distance of two or three sp^3-hybridised carbon atoms from the nitrogen of the ammonium group.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   B01J20/32   B01D15/08   B01J41/06   C12N15/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   B01J   B01D   G01N   C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, EPO-Internal, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

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Date of the actual completion of the international search	Date of mailing of the international search report
22 March 2001	05/04/2001

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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